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Effects of Vertical Transmission of Maternal Antibodies to Mycoplasma Gallisepticum on Growth and Condition of Eastern Bluebird Nestlings (Sialia Sialis)

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EFFECTS OF VERTICAL TRANSMISSION OF MATERNAL ANTIBODIES TO *MYCOPLASMA GALLISEPTICUM* ON GROWTH AND CONDITION OF EASTERN BLUEBIRD NESTLINGS (*SIALIA SIALIS*)

by

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To my two grandmothers, Bobbie Johnson and Jane Denton, for their unwavering love and support in my life, all the while gracefully teaching me the lessons of life best learned outside of the classroom.

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ABSTRACT

Effects of Vertical Transmission of Maternal Antibodies to *Mycoplasma gallisepticum* on Growth and Condition of Eastern Bluebird Nestlings (*Sialia sialis)*

Mothers display maternal effects to influence the development and success of offspring through processes such as resource allocation. In birds, this occurs largely through the egg yolk with the vertical transmission of antibodies to offspring to protect the vulnerable young from disease. Specifically, poultry and eastern bluebirds across the eastern United States are commonly infected by the bacteria *Mycoplasma gallisepticum* (MG), for which mothers may produce antibodies against. To test for the vertical transmission of MG-specific antibodies from mothers to nestlings in eastern bluebirds and the associated fitness effects, bluebird boxes were set up and monitored during the field season. Nests were sampled from over a span of 14 days, and growth measurements and blood samples were taken periodically from nestlings. The serum, taken from nestlings at 2 and 5 days old, was used to run direct enzyme linked immunosorbent assays (ELISAs). We compared nestlings who had and did not have antibodies, and we examined the effects of antibody concentration. A large proportion of adult females tested positive for MG-specific antibodies, and a small proportion of their chicks tested positive for those antibodies, confirming some vertical transmission. Nestlings that tested positive for MG antibodies had a greater change in tarsus length over the nestling period. Of these chicks that tested positive for antibodies, a greater amount of antibodies was associated with greater body condition on day 11. However, of the positive antibody nestlings, their antibodies were shown to have catabolized by day 5, suggesting that these do not last long and may simply be a byproduct of reproduction. Although the presence

of antibodies had a positive effect on tarsus growth during the nestling period, the protective effects from MG antibodies are limited because of their quick degradation.

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INTRODUCTION

In addition to genetic contributions, mothers often impact the phenotype of their offspring through behavioral or physiological influences during embryonic growth and development. Such maternal effects influence a multitude of ecological and evolutionary processes as they are likely to alter fitness outcomes and selective pressures on phenotype (Wolf 2009). One of the principle modes in which females influence the survival of their offspring is by varying the allocation of her resources to progeny. Maternal effects are a product of selection on the mother and offspring. For example, when a mother is experiencing harsh environmental conditions, she may produce offspring that delay development. The mother's position and timing at which the offspring are placed may predict offspring success, sex, and number (Mousseau & Fox 1998). In birds, environmental conditions, such as resource availability, can trigger mothers to invest more or less in yolk carotenoid content (Blount et al. 2002). If the environment is unpredictable, mothers may produce a variability of offspring phenotypes to increase her chances of having successful offspring (Marshall & Uller 2007).

Species within the avian order Passeriformes, which includes songbirds, produce altricial young. Altricial birds hatch lacking an adaptive immune system, thus they are unable to generate their own protective antibodies when they are at their most vulnerable. One potentially beneficial effect that non-mammalian mothers can have on their developing offspring is to deposit antibodies, a vital part of the immune system, into their eggs prior to laying (Boulinier & Staszewski 2008). This vertical transmission has been found to occur in precocial chickens, which are born more developed than altricial song birds, so this serves as a foundation for studying the same effect in songbirds (Hartle 2014).

Humoral Immunity

The immune system is a functional system that protects the body from incoming pathogens by implementing two independent but coordinated systems—the innate and adaptive systems. The innate immune system is nonspecific and prevents the entry of the pathogen, whereas the adaptive immune system includes an antigen-specific immune response and immunological memory. Antigens are non-self substances that elicit an immune response. One side of the adaptive immune system is humoral immunity, which involves antibodies. My research was primarily focused on the humoral immune response of the adaptive system. Once a naive B lymphocyte is activated by binding an antigen, clonal selection occurs. The B cell grows and multiplies into a clone, or group, of cells that have the same antigenic receptors as itself. Most cells in the clone turn into plasma cells, which secrete large amounts of antibodies. Antibodies are blood proteins that are produced to counteract antigens. These antibodies circulate throughout the body, where they can bind to antigens and target them for destruction. Other clone cells become memory cells, which work to mount quicker future responses to the same antigen by providing immunological memory. Upon subsequent exposure to the same antigen, immune memory provides quicker, longer, and more effective responses (Marieb & Hoehn 2007).

Benefits of Maternal Transmission of Antibodies

In mammals, antibodies may be transferred pre- and postnatally through the placenta and milk, respectively (Boulinier & Staszewski 2008). In avian species, IgM is the predominant immunoglobulin, or antibody, produced during the primary humoral response (Hartle 2014). During the secondary humoral response, avian IgY is the predominant antibody produced. Avian IgY is the equivalent of human IgG. IgY is the particular immunoglobulin that is transmitted prenatally from the mother's circulation to the egg yolk (Hartle 2014). Because the humoral immune system is not yet fully developed in newborn nestlings, antibodies produced by the mother's immune system can provide passive protection when transmitted to offspring during egg development. IgY antibodies are transported and absorbed from the yolk sac to the embryo's circulation and later the hatched nestling (Hartle 2014).

In birds, mothers in better condition transmit a higher concentration of yolk antibodies, whereas mothers with high stress transmit less (Hargitai et al. 2006). Female and offspring condition; genetic factors; and environmental factors such as offspring rank, pathogen exposure, resource availability, and egg color are suspected to also cause variation among mothers in antibody transmission (Boulinier & Staszewski 2008). Furthermore, mothers may allocate antibodies in different ways to different eggs since they lay a single egg per day until clutch completion. In some bird species, since the youngest nestling is at a disadvantage, mothers allocate more immunoglobulins to it to increase its chances of success (Hargitai et al. 2006). In other bird species, for brood reduction, antibody concentration decreases as the eggs are laid (Hargitai et al. 2006).

The transmission of maternal antibodies may benefit offspring by causing earlier maturation and fledging (Hasselquist & Nilsson 2009). Offspring with a higher concentration of maternal antibodies have higher growth rates, and those with lower levels have retarded growth (Grindstaff et al. 2003). At too high levels, however, these may be harmful by having blocking effects, preventing the offspring from developing active immunity of their own (Grindstaff et al. 2003). My research focused on the effects of transmission of protective antibodies against the common pathogen, *Mycoplasma gallisepticum,* in eastern bluebirds.

Mycoplasma gallisepticum

Mycoplasma gallisepticum lacks a nucleus and a cell wall, which contributes to its shape, antibiotic resistance, and nutritional needs (Kleven 2003). Instead, each cell is surrounded by a plasma membrane, which is made up of proteins that act as surface antigens and contribute to the bacteria's adhesion properties (Fischer et al. 1997, Levisohn & Kleven 2000, Ley 2003). These factors allow the bacteria to evade the immune system and adapt to their hosts' environments to infect cells. *Mycoplasma gallisepticum* (MG) is a gram-negative bacteria that can cause acute and chronic disease of poultry species, causing symptoms such as respiratory rales, conjunctivitis, nasal discharge, and coughing (Ley 2003). MG can be horizontally spread to other birds by direct or indirect contact with the infected host (Levisohn & Kleven 2000). Before the shell is laid down, vertical transmission may also occur from naturally infected chickens and turkeys to their eggs while still in the oviduct (Ley 2003). This demonstrates a protective mechanism that mothers may have on their offspring.

In the mid 1990s, MG underwent a host shift from poultry to songbirds, such as house finches, quickly spreading through the eastern population of house finches in North America (Farmer et al. 2005). MG was shown to have a dramatic detrimental effect on house finches' health, causing conjunctivitis (Farmer et al. 2005). This caused strong selection, resulting in evolutionary changes. If MG antibodies do protect offspring from MG, we would expect to find maternal transmission of antibodies to offspring. There is no evidence provided in the primary literature that any songbird host, as opposed to poultry hosts, transmits antibodies to MG through the egg. Likewise, no one has established, if they are transmitted, whether they actually benefit the offspring and how long these antibodies last post-hatch. We can, however, speculate that these processes are occurring.

Eastern Bluebirds

The eastern bluebird is a medium-sized thrush (Family, Turdidae) common across the eastern half of the United States (Figure 1). Bluebirds are secondary cavity nesters; they breed in natural tree cavities, including those made by woodpeckers. Like many secondary cavity nesters, they often nest in man-made nestboxes when natural cavities are scarce (https://birdsna.org/Species-Account/bna/species/easblu/). As a result, they make for ideal study organisms when studying a wild population because they are easily monitored for the length of the breeding season (approximately six months).

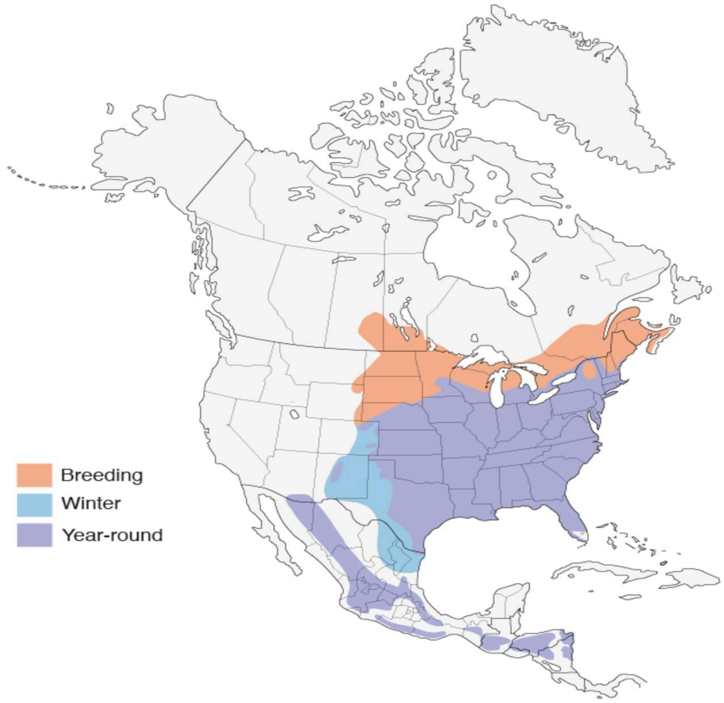

Figure 1. Distribution of eastern bluebirds across North America. (Image taken from www.allaboutbirds.org).

Nest building begins in late February or early March, and the peak breeding month is April. The females build the nests as a loose cup made up of grass, pine needles, and feathers. Egg-laying begins once the nest is finished with mothers laying 1 egg each morning. Clutch size ranges from 3-7 eggs and varies by latitude. Incubation by the female begins once the last egg is laid and lasts approximately 14 days. Many eggs hatch early in the morning, but the duration of hatching varies from 1-6 hours. The newly hatched young require extensive parental care in the form of biparental feeding and maternal incubation. Females brood to give the nestlings warmth until they are 5-7 days old. By the end of day 1, stereotypic begging posture is achieved by the young. Wings lengthen and feathers develop over the next two weeks, becoming completely feathered by day 15. Fledging age varies depending on the location and resources, but it typically occurs around days 17-19. The number of broods reared per season may vary, but two broods per year is most common (https://birdsna.org/SpeciesAccount/bna/species/easblu/).

Preliminary data

In the summer of 2017, the Balenger lab conducted a preliminary study on eastern bluebird adults and nestlings and the prevalence of MG and associated antibodies. MG has been found to be a very common infection across the eastern bluebird population. Upon catching the wild adult bluebirds, about 36% of them tested positive for MG-specific antibodies, which indicated a current or prior MG infection. This prompted a closer look at their young. A tube of blood was taken while the nestling was around 6-11 days old, and a Serum Plate Agglutination (SPA) assay was used to assess the presence of MG-specific antibodies. During an SPA, a drop of blood is combined with the antigen. If the antibody is present in the nestling's blood, agglutination is visible as the antibody binds with the antigen. It was found that about 15% of nestlings belonging to MG antibody positive mothers also had MG antibodies during this time period; however, the sample size was small, including 39 nestlings from 13 nests. The presence of these MG-specific antibodies means that some vertical transmission was occurring.

Hypotheses and predictions

My study asks whether female eastern bluebirds (*Sialia sialis*), a common North American songbird, 1) transmit antibodies via their eggs that persist in small, altricial nestlings, and 2) whether these antibodies affect aspects of nestling quality and fitness. Specifically, I asked whether the presence of antibodies specifically targeted to protect against a common bacterial pathogen (*Mycoplasma gallisepticum*) was associated with larger, faster growing young that were more likely to successfully fledge from the nest. Such an association would suggest that maternal antibody transmission improves fitness and, thus, is adaptive in eastern bluebirds.

To test whether female eastern bluebirds transmit protective MG-specific antibodies to their offspring, I asked to what extent nestlings of MG antibody positive mothers test positive for these same antibodies. I expected that any MG antibody present in nestlings at 2 days old must have been transmitted from the mother, since nestlings would still be too young to produce their own antibodies. I asked how long post-hatch these antibodies persist. I predicted that if antibodies provide true disease protection, they will persist at least until nestlings are 5 days old, while they are still unable to produce their own antibodies. Finally, if nestlings with antibodies are receiving a benefit, I predicted that they will grow faster and be in better condition at fledging than those without antibodies.

METHODS

Data collection

Bluebird boxes with predator guards were set up 100 meters apart at the University of Mississippi Field Station (UMFS) and the University of Mississippi Golf Course (UMGC). There were 55 boxes at the field station and 26 at the golf course. In 2018, bluebird boxes were observed every 1-3 days, usually in the morning or early afternoon, from March until the end of July. Observation included watching the birds in the area and looking inside the bird box. Notes were taken regarding the nest stage, number of eggs, number of nestlings, nestling ages, and presence of adult birds. Hatch day was designated day 0 of the nestling's life. On days 2, 5, 8, 11, and 14 in the nestling's life, the young were sampled (Figure 2). All animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of the University of Mississippi (IACUC protocols #18-015).

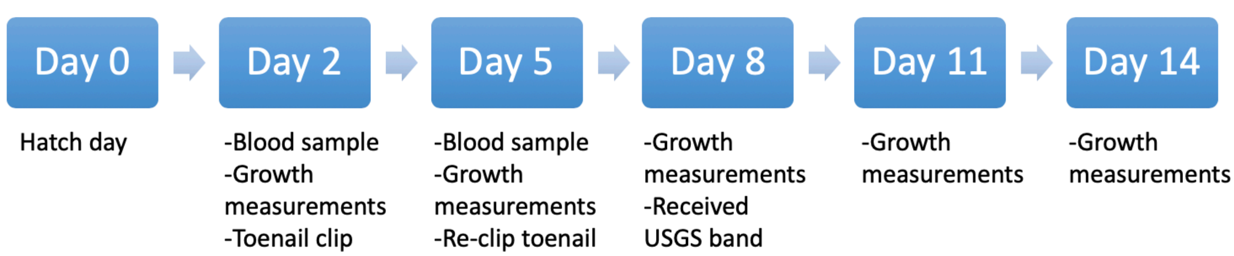

Figure 2. Timeline of field work done on the bluebird nestlings.

Growth measurements included weighing the nestling and measuring its tarsus bone length. The toenail was clipped for future identification so that the young could be banded once they were old enough. On day 2, blood was taken from the nestling's jugular vein, but on the other days, blood was taken from the brachial vein. Water drops were given to nestlings before and after drawing blood to keep them hydrated. As recommended by the Ornithological Council, no more than 1% of the bird's body weight was taken when drawing blood (Fair et al. 2010). To take blood, the vein was pricked using a sterile needle. A capillary tube was used to draw up the blood droplets, and then the blood was transferred to a micro centrifuge tube. The blood was kept on ice. Upon returning to the lab, samples were centrifuged to separate red blood and serum. Serum was pipetted off the top and placed into a different tube. Samples were kept at -28°C until tested for antibodies. Adults were caught when their nestlings were approximately 5-11 days old. An observer was present at all times during trapping. To trap adults, a piece of plastic cellophane was taped on the inside of the nest box so that it covered the entrance hole. Once the adult flew into the box to feed nestlings, the plastic would work like a flap to trap the adult bird. The box was then opened, and the adult bird was taken from the house. Size measurements and a brachial vein blood sample was taken from the adult. The same process was used for adult blood samples to separate the serum into a separate tube. The bird was given a silver government issued USGS leg band as well as three colored leg bands for easy identification in the field.

Enzyme Linked Immunosorbent Assay (ELISA)

The serum was used to run a direct enzyme linked immunosorbent assay (ELISA), specifically using the commercially available IDEXX MG kit. This assay was used to detect the relative concentration of MG-specific antibodies in the serum of eastern bluebird mothers and their nestlings. There were 35 families that were sampled, and 30 of these females were tested for MG-specific antibodies. All day 2 nestlings were tested for antibodies. Day 5 serum was only tested for nestlings that showed evidence of maternal antibody transmission, such as those that had the antibodies at day 2 or whose mother tested positive for antibodies. Plates were pre-coated with MG antigen. A blocking step was initially applied for 40 minutes using a 1:10 dilution of 1% bovine serum albumin in phosphate-buffered saline to prevent unwanted binding of the antibody to the plate itself. Then, the wash step consisted of washing the plate three times with the solution made up of Phosphate-buffered saline (PBS) and Tween 20. The serum samples were thawed on ice and centrifuged briefly. Sample diluent provided with the kit was used to dilute serum samples 1:50. We tested three controls: blank, negative, and positive. Samples and the controls were dispensed in duplicate into wells. The plate was incubated for 1h to allow the antibody in the serum to bind to the antigen coated on the wells. Then, the wash step washed away any unbound antibody. Conjugate, (Goat) anti-chicken horse radish peroxidase, was dispensed into each well, which bound to attached antibody in the wells. The plate incubated for 1h and was washed again to remove unbound Conjugate. Enzyme substrate, Tetramethylbenzidine, was added to each well to bind to the conjugate and cause a color change. The extent of color change was related to the amount of antibody present in the sample. After a final 15-minute incubation, stop solution was pipetted into each well to stop any additional binding.

Data Analysis

The plate was placed into a Biotek EL x 800 Universal Microplate Reader, and absorbance values were measured and recorded at 630 nm. This allowed for the calculation of an ELISA value for each sample by using the mean of the sample duplicates. The amount of antibody could then be quantified by categorizing samples as positive or negative for MG-specific antibodies. This was determined using an approximate ELISA cut-off value of 0.0229, which was previously designated for experimental study of house finches and domestic canaries using the same ELISA kit (Hawley et al. 2011).

Statistical analyses were performed to examine the relation between the presence or absence of MG-specific maternal antibodies in eastern bluebird nestlings and several fitness indices including changes in growth and body condition throughout the two-week sampling period. The growth rates of nestlings were quantified by the difference in mass between day 14 and day 2. Body size was estimated by measuring tarsus length. For adults, the tarsus bone is used because it becomes a fixed value as they age. In nestlings, this elongated ankle bone changes in length over time, so it is a good indicator of nestling growth. Body condition was calculated for each sampling time point from the residuals of regressions between mass vs tarsus length. To examine relationships between nestling body condition, growth and the categorical presence or absence of MG-specific antibodies, a Kruskal-Wallis test was performed. To explore relationships between numerical ELISA antibody units and nestling body condition and growth, Spearman rank correlations were used. Both Spearman rank correlations and Kruskal-Wallis tests were chosen to account for our non-parametric data due to a small sample size.

RESULTS

Sample size varied slightly between field sites (Table 1). MG-specific antibodies were found to be very prevalent among adult female bluebirds; however, these maternal antibodies were present only in a small number of nestlings on day 2 (Table 2). Of the day 2 nestlings that tested positive for MG antibodies, none of these nestlings remained positive by day 5, indicating that the antibodies were catabolized between days 2 and 5.

Effects of the Presence of MG Antibodies

When examining all nestlings, the presence vs absence of MG antibodies did not affect body condition or mass change (Figure 3, Figure 4). However, the presence of MG antibodies was found to have a significant effect on the change in tarsus length over the nestling period. Specifically, nestlings that were positive for MG antibodies had a greater change in tarsus length in comparison to nestlings that were negative for MG antibodies ($p=0.007$) (Figure 4).

Effects of the Amount of MG Antibodies

Among nestlings that tested positive for MG antibodies, those with the highest concentration of antibodies also were in the best body condition at 11 days old, but not on any other day sampled (Table 3). Furthermore, the difference in mass and tarsus length between 2 day old and 14-day old nestlings was not significantly correlated with MG antibody concentration (Table 3).

Table 1. Summary of sample sizes at each field site utilized in this study. UMFS = University of Mississippi Field Station; UMGC = University of Mississippi Golf Course

	UMFS	UMGC
Nests sampled	20	15
Adult females sampled	17	13
Nestlings sampled	73	45

Table 2. Summary of antibody testing results. Percent refers to the percentage of individuals testing positive relative to the entire number of individuals tested.

	n	%
Positive adult females	21	70
Positive 2 d. o. nestlings	10	8
Positive 5 d. o. nestlings	0	0

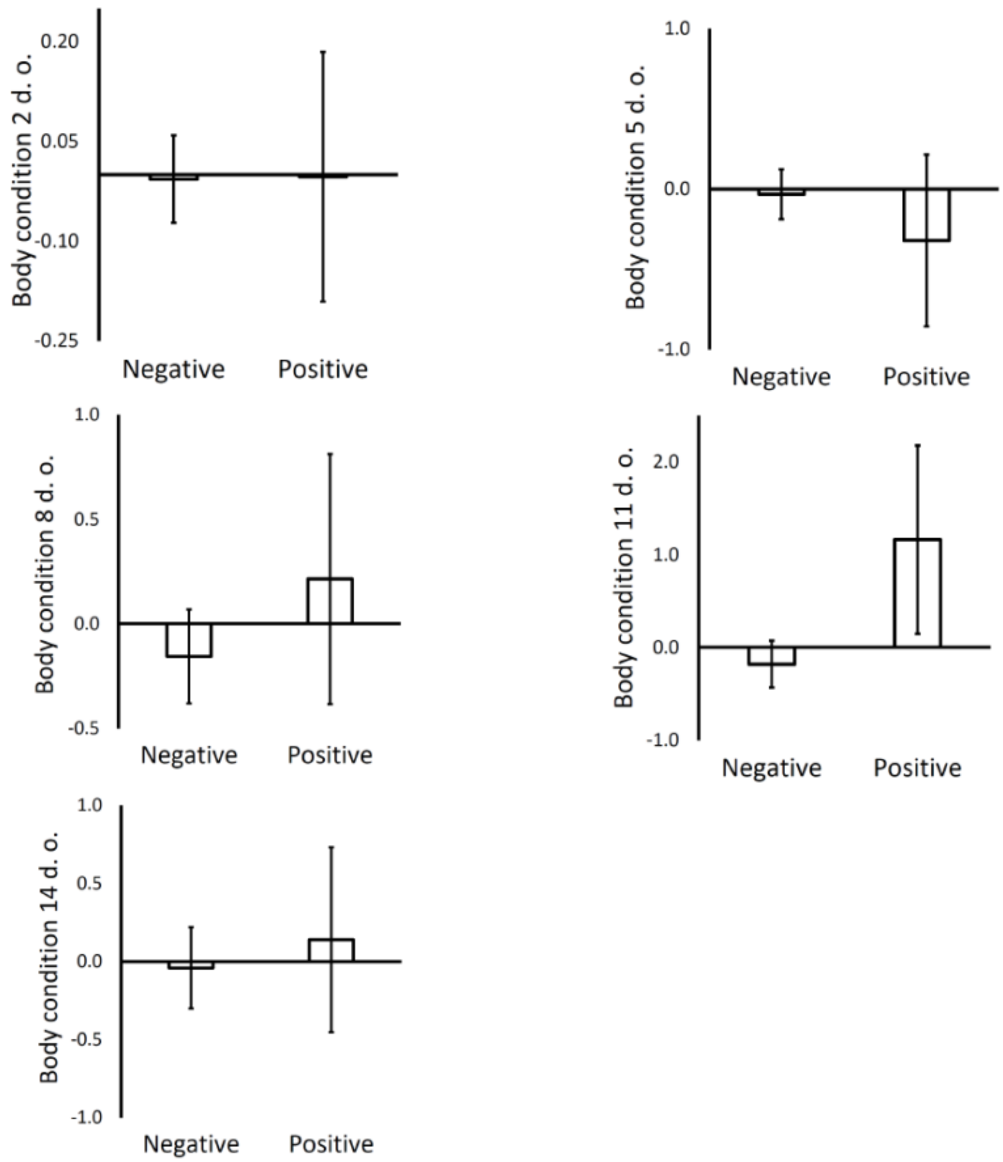

Figure 3. Effects of the presence or absence of MG antibodies on body condition at 2, 5, 8, 11, and 14 days old. Plots illustrate means (n=118) with standard error bars.

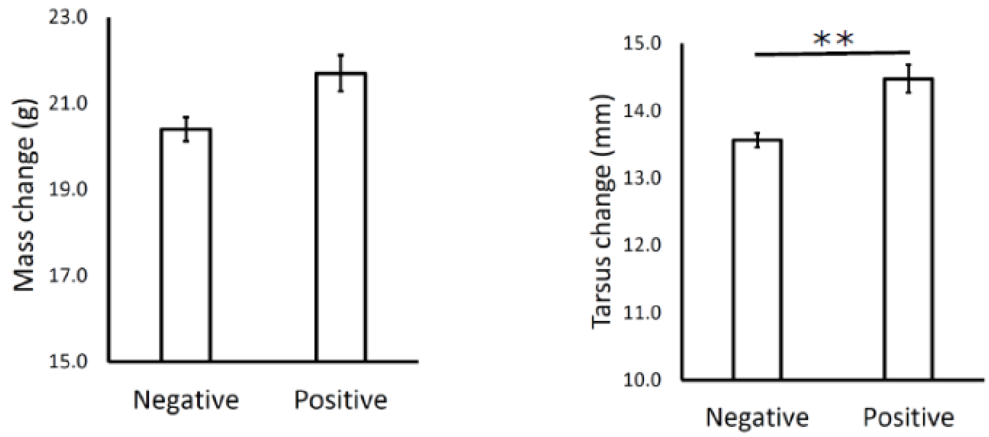

Figure 4. Effects of the presence or absence of MG antibodies on mass change (g) and tarsus length change (mm) between the ages of 2 and 14 days old. Plots illustrate means (n=118) with standard error bars.

Table 3. Spearman rank order correlations of individuals testing positive for MG-specific antibodies. Each column shows the effect size (*rho*), associated *p*-value, and sample size. Significant *p*-values are bolded.

	Body condition						
	2 d. o.	5 d. o.	8 d. o.	11 d. o.	14 d. o.	Mass change	Tarsus length change
Nestling antibodies (rho)	0.520	0.367	0.597	0.672	0.269	-0.042	-0.008
p-value	0.124	0.297	0.090	**0.047**	0.484	0.915	0.983
n	10	10	9	9	9	9	9

DISCUSSION

Since chicks that were positive for MG-specific antibodies on day 2 were negative for antibodies by day 5, there may not be much of a protective effect conferred to nestlings because of this quick degradation. Disease-specific, maternally-transmitted antibodies may be limited in their ability to extend immunity against MG. If the mother passes the disease as well as the antibodies to the nestling through the egg, then the antibodies may provide a protective effect while the nestling is still in the egg and shortly after hatching. This is supported by an experiment done on chicken eggs. During an experiment in which 7-day old chicken eggs were inoculated with MG through the yolk sac, embryos died within 5-7 days (Ley 2003). Interestingly, eggs that contained MG antibodies from the mother were more likely to avoid mortality (Ley 2003). These antibodies may also positively contribute to hatching success. When a small percentage of eggs fail to hatch, this may be indicative of an infection by MG or lack of antibodies. My study could be further developed by examining unhatched eastern bluebird eggs to test for the presence of MG-specific antibodies.

These maternal antibodies may help to prime the immune system of the nestlings. There was only a single time point in which body condition was found to be significantly affected by the concentration of antibodies (day 11). By day 11, the nestlings are able to mount their own immune response, so it is unclear how much the concentration of maternal antibodies on day 2 would affect body condition on day 11. However, it has been found that pied flycatcher nestlings that came from immunized mothers had an increased antibody production (Grindstaff et al. 2006). This could be attributed to the fact that the initial transmission of maternal antibodies to nestlings primed their immune systems to positively influence their ability to produce antibodies later (Grindstaff et al. 2006). This enhanced ability to produce antibodies later may lead to better body condition.

The increased tarsus change that is associated with the presence of MG antibodies could be beneficial to nestlings to promote earlier fledging from the nest. In humans, maternal effects are experienced through the breast milk. Specifically, mothers transmit antibodies to their babies as well as growth factors that stimulate cell development and overall growth (Cacho 2017). It is possible that similar to this maternal effect in humans, eastern bluebird females could transmit growth factors and other elements along with antibodies to stimulate growth in the nestlings. This may explain why the presence of MG antibodies is associated with a faster growth in the tarsus bone.

To our knowledge, this is the first study to investigate the vertical transmission of MG-specific antibodies in a wild songbird. The idea that maternal antibodies are degraded by day 5 and may not confer much immune protection is consistent with findings on altricial house sparrows, which are in the avian order *Passeriformes* as well (King 2010). In this study, DNP-KLH-specific antibodies were absent 3 days post-hatch in house sparrow nestlings, having a half-life of 2.2 ± 0.25 days, suggesting that mothers may not provide as much immunological protection as thought previously (King 2010). Immunologic independence was found to occur approximately 10 days post-hatch in both altricial house sparrows and precocial chickens, at which point maternal antibodies are no longer detected and the chicks generate their own antibodies (Hartle 2014, King et al. 2010). A study done on pied flycatchers found the same rapid decline in maternal antibodies a few short days post-hatch (Grindstaff et al. 2006). These findings seem to be consistent with the data from my study. The pied flycatcher study also found that antibody levels increased between days 5 and 14, demonstrating the nestlings beginning to produce their own antibodies, which is consistent with the immunological independence time period found in chickens and house sparrows (Grindstaff et al. 2006, Hartle 2014, King et al. 2010).

Maternally transmitted MG antibodies confer somewhat of a protective effect and help facilitate growth. Data does not support that the antibodies alone contribute to greater growth among nestlings because of the presence of other contributing factors such as the availability of food, weather conditions, or extent of parental care. The post-hatch environment has significant effects on the growth and condition of the nestling. This was confirmed from a study done on wild great tits, in which nestling growth was found to be greatly affected by rearing conditions that post-hatch nestlings encountered (Martyka et al. 2018). They found that overall, those in harsher post-hatch conditions grew slower versus those under ideal conditions (Martyka et al. 2018).

The concentration of antibodies does not appear to have as much of an effect as the general presence of antibodies does, so more antibodies may not necessarily be providing further benefit to offspring. It may be that adult females benefit the most from transmitting just enough antibodies to protect her young but not an excessive amount to the point of depleting her own antibody reserve. The concentration of antibodies allocated to eggs may be affected by the concentration of antibodies the mother has. In a study done on collared flycatcher eggs, mothers with a higher concentration of antibodies were found to allocate more antibodies to the yolk of her young (Hargitai et al. 2006).

It is evident that the presence of antibodies does confer some beneficial effect to nestlings since antibody positive nestlings had a greater tarsus change over the nestling period, which means that they are growing faster. Since the vertical transmission of antibodies is indeed occurring in eastern bluebirds, it is assumed that this process is not harmful to the mother, or else it would be selected against. This does not mean that there are not costs and benefits for the mother, though. In a study on chickens, mothers were found to lose 10-20% of their own antibody level per egg (Kowalczyk et al. 1985). These precocial mothers stimulate their own immune systems to make more antibodies and account for the loss, which comes with energetic and nutritional costs (Kowalczyk et al. 1985).

It is still possible that vertical transmission is only occurring as a byproduct of reproduction and does not provide protective effects to offspring post-hatch. While it seems reasonable to hypothesize that MG-antibodies may provide protection to developing embryos, especially within the egg, my study has shown that any benefit to nestlings is limited since the antibodies have been shown to be depleted by day 5, at which point the nestlings are still unable to mount their own immune response and are still highly susceptible to pathogens.

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